Nutrient Supply and Mycorrhiza Development in Caribbean Pine

BY
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Abstract. Changes are reported in mycorrhiza development of *Pinus caribaea* Mor. seedlings, following the addition of nutrient salts; the plants were raised in the greenhouse in topsoil collected from a young pine plantation. Problems of assessing mycorrhiza frequency are discussed, and sampling procedures described. Treatments were compared on the basis of (a) the number of infected seedlings expressed as a percentage of the total number of seedlings per pot, and/or (b) "mycorrhiza percent," defined as the number of mycorrhizae expressed as a percentage of the total number of short roots per seedling. The presence of dichotomous branching or forking of the short roots was taken as the criterion of mycorrhizal infection. Mycorrhiza development was reduced by increasing the level of soil nitrate, either by adding ammonium nitrate or by liming, but was unaffected by change in soil reaction per se. An inverse, nonlinear relationship was found to exist between the total nitrogen content of seedling roots and mycorrhiza percent (multiple correlation coefficient $R = 0.81$). The addition of sodium dihydrogen phosphate lowered the percentage of nitrogen in the roots, and increased mycorrhiza development. A test was made of Björkman's hypothesis that the formation of mycorrhizae depends on soluble carbohydrate being present in the roots. Mycorrhiza percent was correlated with the ratio of soluble carbohydrate (percentage reducing sugars) to total nitrogen ($r = 0.72$) but not with the level of soluble carbohydrate alone ($r = 0.13$).

HARLEY (1952) expressed the opinion that, under natural conditions, mycotrophic tree species obtain most if not all of their nutrients by means of mycorrhizae. If this is so, then a study of the factors governing the formation and development of mycorrhizae is important, both for practical and theoretical purposes. This paper reports the effects of changes in the level of certain nutrients on the degree of mycorrhizal infection in seedlings of caribbean pine (*Pinus caribaea* Mor.).

Several workers (Hatch 1937, Björkman 1942, 1949, Fowells and Krauss 1959, Hacskaylo and Snow 1959) have shown that an inverse relationship exists between nutrient availability and the degree of mycorrhizal infection in *Pinus*. Hatch (1937) considered that the predisposing factor in mycorrhiza formation was plant nutrient status, especially that of nitrogen, phosphorus and potassium: where the internal concentrations of these nutrients were high and properly balanced, few my-

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corrhizae would form. Björkman (1942), however, concluded that the production of mycorrhizae was conditioned primarily by the presence of free soluble sugars in the roots.

Materials and Methods

Details of pot culture methods have been given elsewhere (Richards 1961). The closed pot system was used, and the growth medium was the A_1 horizon of a sandy podzolic soil, collected from a young pine plantation at Beerburrum in southeast Queensland. Polyethylene pots 7 inches in diameter, containing approximately 3 kg of fresh soil, were used, and nutrients applied on a weight per unit area basis (1 cwt[1] per acre = 0.275 g per pot).

Total nitrogen content of plant tissue was determined by a micro-kjeldahl technique, modified to include nitrate. The method used for analysis of soluble carbohydrate (reducing sugars) followed Plank's (1936) modification of Harding and Downs' technique.

Soil pH was measured with the glass electrode in a 1:5 soil-to-water suspension after mechanical shaking for 1 hour, on samples taken from the pots with a cork borer. Nitrogen fractions were determined on soil cores extracted likewise; ammonium nitrogen was analyzed by nesslerization and oxidized nitrogen (nitrite + nitrate) by the brucine test, as described by Peech and English (1944). Nitrite was measured by the method of Rider and Mellon (Barnes and Folkhard 1951).

Assessment of mycorrhiza development. Several kinds of mycorrhizae are recognized in *Pinus*, each with a characteristic morphology. The forked mycorrhizae of the seedlings examined in this study were predominantly of Melin's type *A* and occurred, as described by Robertson (1954), in acropetal succession on the parent long roots; type *B* mycorrhiza were far less common. Isolation and identification of the mycorrhizal fungus was not possible; however, Young (1940) found that both *Boletus granulatus* L. ex Fr. and *Rhizopogon rubescens* Tul. (syn. *R. roseolus* Dodge) were common mycorrhiza formers with *Pinus* in this region.

Comparison of mycorrhiza development may be made in a number of ways. Since mycorrhizae are nutrient absorbing organs it would seem logical to describe their development in terms of those features which are concerned in the process of nutrient uptake. For example, if prolific forking increases the efficiency of mycorrhizae for absorbing nutrients, then the extent of forking should be taken into account when assessing treatment effects (see Hatch 1937). However, until the functional aspects of the various kinds of mycorrhizae are better understood, comparisons of mycorrhiza development must remain on an empirical basis. In the present study, comparisons are based on (1) the number of infected seedlings expressed as a percentage of the total number of seedlings per pot, and/or (2) the number of mycorrhizae expressed as a percentage of the total number of short roots per seedling ("mycorrhiza percent"). The criterion of mycorrhizal infection was the presence of dichotomous branching or forking of the short-roots. Since dichotomy in short roots of *Pinus* is not always associated with mycorrhizal infection (Hatch and Doak 1933, Levisohn 1954, Slankis 1958), some care is necessary in accepting forked short roots as mycorrhizae. In the studies reported here, counting was carried out under a binocular microscope (12.5×), and after experience had been gained in this way a binocular loupé was used. No distinction was made between types *A* and *B* mycorrhizae. Where any doubt existed as to their mycorrhizal status, the short roots were regarded as uninfected. (This was the position with unbranched mycorrhizae: while these could frequently be recognized under the microscope, their identification with the binocular loupé was by no means certain).

Counting the individual short roots on a large number of seedlings is tedious. Hatch (1937) made such complete enumerations in his preliminary experiments in Sweden; however, in his 1931 experiments in the U.S.A., he made a visual estimate of the percentage of mycorrhizae on each seedling. Such an ocular estimate was apparently the basis for his calculation of "mycorrhizal points" in subsequent experiments; at least he did not clearly specify otherwise. Early in the investigations reported here, an attempt was made to assess mycorrhiza development by ocular estimate. A system outlined by Horsfall (1945) for grading plant disease was tested both for entire root systems and for individual long roots, but in neither case could the results obtained be correlated with those from actual counts. Ocular estimates were therefore discarded in favour of complete enumeration of a sample of the whole population.

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[1] 1 cwt = 1 hundredweight or 112 lbs.

Sampling procedures. Certain factors complicate the choice of a sampling system. Unless all short roots have equal opportunity for infection even a random sample may be biased. Granting Robertson's (1954) contention that the mycelium ramifying in the cortex of the long root is a common source of infection for emerging short roots, then short roots borne on infected long roots would have greater opportunity of becoming mycorrhizae than those carried by uninfected long roots. Therefore estimates based on unrestricted random sampling of long roots from any one seedling are likely to be of low precision. This would be particularly important in situations where infection foci in the soil are limited in number, but is probably of lesser significance in the experiments described here where the growth medium was surface soil taken from a vigorous pine plantation. The estimate of mycorrhiza percent in these experiments was based on a complete enumeration of the root system of one seedling selected at random. Where the root system thus chosen was felt to be definitely atypical, a second was taken also at random, both were enumerated and the results averaged: this procedure increases the precision of the estimate but does not bias the sample. In Experiment 1, where not all the seedlings were mycorrhizal, the sample was drawn from infected seedlings only; mycorrhiza percent for each pot was then calculated from the expression $\frac{pn}{N}$ where p is the percentage of forked roots on the seedlings sampled, n is the number of infected seedlings and N the total number of seedlings per pot.

Description of Experiments and Presentation of Results

Experiment 1. This trial was conducted in partially sterilized soil, and the effects of nitrogen, phosphorus, potassium, calcium, copper, iron and inoculation with fresh soil were studied. The choice of nutrients was dictated by the results of previous experiments with loblolly pine. The purpose of the higher of the two levels of lime used was to raise soil pH; the lower level was primarily to test the effect of calcium as a nutrient. The design was a 2^6 factorial, duplicated in randomized blocks, and the following treatments were applied:

N as ammonium nitrate, nil or 3 cwt per acre;
P as sodium dihydrogen phosphate, 0.43 or 2.58 cwt per acre;[2]
K as potassium chloride, nil or 1 cwt per acre;
Ca as calcium carbonate, 4 or 20 cwt per acre;
Cu as cupric chloride, nil or 7 lb per acre, and Fe as Fe-EDTA, nil or 2.8 lb Fe per acre as a combined dressing;
Inoculation, nil or achieved by replacing about 25 grams of soil with an equal quantity of fresh soil from a 4-year old plantation of caribbean pine.

The pots were sown to *P. caribaea* on Februray 13, 1957 using seed which had been surface sterilized by washing in 0.1

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[2] Equivalent in phosphorus content to 1 or 6 cwt per acre of superphosphate, respectively.

percent solution of mercuric chloride. The experiment was harvested on July 3, 1957 and the results are shown in Table 1. Yield data reflect the effects of treatment on germination and mortality as well as on growth, since no thinning was done; these effects will be dealt with elsewhere. Increasing the level of lime from 4 to 20 cwt per acre promoted nitrification of the ammonium ion. The NxCa interaction for ammonium nitrogen was highly significant ($p<0.01$) due to a decrease in ammonia at N_3Ca_{20}. There was a corresponding increase in nitrate at N_3Ca_{20}, although the NxCa effect was here not quite significant.

The root systems of all seedlings in each of the 132 pots were examined at harvest, and the number showing evidence of mycorrhizal infection recorded; this was expressed as a percentage of the total number of seedlings per pot, and provided an additional means of comparing mycorrhiza development. Mycorrhiza percent was determined only for the 32 pots which made up a factorial combination of N, Ca, P and inoculation (2 replicates). Since the experiment was harvested at an early stage of mycorrhiza development, the degree of mycorrhizal infection is probably best reflected by the proportion of the total number of seedlings in each pot which were infected. Some seedlings growing in partially sterilized soil became mycorrhizal, and this may have been due either to reinfection by air-borne spores, as described by Robertson (1954), or to ineffective sterilization.

The roots of seedlings growing under treatments which produced high levels of soil nitrate had a high total nitrogen content and relatively few mycorrhizae. For example, the percentage of mycorrhizal seedlings was least at N_3Ca_{20} and greatest at N_0Ca_4, thus: N_0Ca_4, 41.6%; N_0Ca_{20}, 20.3%; N_3Ca_4, 10.8%; N_3Ca_{20}, 4.0%. The corresponding values for the nitrogen content of roots were N_0Ca_4, 1.02%; N_0Ca_{20}, 1.48%; N_3Ca_4, 2.36%; N_3Ca_{20}, 2.57%.

Sodium dihydrogen phosphate produced more mycorrhizae at 2.58 cwt per acre (P_6) than at 0.43 cwt per acre (P_1). The greatest percentage of mycorrhizal seedlings was found at N_0P_6 and the least at

TABLE 1. Effect of NH_4NO_3, $CaCO_3$, $NaH_2PO_4 \cdot 2H_2O$ and inoculation with fresh soil on ammonium and nitrate levels in the soil, nitrogen content of roots, yields, and mycorrhiza development of Pinus caribaea *in partially sterilized soil (experiment 1).*[1]

Treatment	NH_4-N 20 March 1957[2]	NO_3-N 20 March 1957[2]	Total N roots	Dry weight whole plant	Mycorrhiza development[3] Infected seedlings[4]		Mycorrhiza percent	
	ppm	*ppm*	*percent*	*g*	*percent*			
N_0	22.5	19.8	1.25	3.92	31.0	(29.51)	1.9	(5.98)
N_3	50.7	51.8	2.46	1.60	7.4	(7.78)	0.4	(1.51)
Ca_4	50.7	22.4	1.69	3.68	26.2	(24.61)	1.8	(5.16)
Ca_{20}	22.5	49.2	2.02	1.84	12.2	(12.68)	0.5	(2.33)
P_1	n.d.[5]	n.d.	1.96	2.45	12.3	(12.61)	1.2	(3.49)
P_6	n.d.	n.d.	1.74	3.07	26.1	(24.68)	1.1	(4.00)
Uninoculated	41.6	40.2	1.90	3.18	6.4	(7.87)	0.3	(1.55)
Inoculated	31.6	31.4	1.82	2.34	32.0	(29.42)	2.0	(5.94)
Sig. Diff.								
($p<0.05$)	9.2	17.1	0.16	0.28		(4.44)		(2.71)
($p<0.01$)	13.4	24.4	0.23	0.38		(5.87)		(3.65)

[1] Yield and percentage of infected seedlings, means of 64 pots; "mycorrhiza percent," means of 16 pots; plant and soil nitrogen, means of 8 pots. Subscript numerals represent treatments in cwt/acre (P as superphosphate equivalent).

[2] Five weeks after sowing, 15 weeks before harvest.

[3] Mean arc sine transformations shown in brackets.

[4] Percentage of total number of seedlings per pot.

[5] n.d. = Not determined.

TABLE 2. Effect of NH_4NO_3 and $CaCO_3$ on soil nitrate level, soil pH, and yield, nitrogen content and mycorrhiza development of Pinus caribaea *(experiment 2).*[1]

	Treatment				Sig. Diff.	
	N_0Ca_0	N_0Ca_{16}	N_3Ca_0	N_3Ca_{16}	$p<0.05$	$p<0.01$
NO_3-N (ppm)[2]						
30 April 1957	7.4	7.2	72.4	66.3	12.9	17.4
10 June 1957	12.7	21.3	71.4	115.3	12.3	16.7
19 November 1957	3.2	3.1	8.2	59.7	22.2	24.6
Dry Weight (g)						
Shoots	4.40	5.00	5.80	3.33	0.91	1.26
Roots	2.78	2.61	2.29	1.35	0.43	0.59
Shoot/root ratio	1.63	1.94	2.53	2.47	0.15	0.21
Soil pH						
19 November 1957	6.3	7.5	5.8	6.9	0.2	0.3
Total N-Shoots						
Actual data (%)	0.99	1.18	2.03	2.44		
Transformed data[3]	5.66	6.19	8.14	8.94	0.43	0.59
Total N-Roots						
Actual data (%)	0.83	1.02	1.70	2.46		
Transformed data[3]	5.23	5.81	7.50	9.00	0.49	0.67
Mycorrhiza percent						
Actual data	19.5	8.7	2.0	4.3		
Transformed data[3]	25.83	16.86	7.21	8.19	5.26	7.25

[1] Means of 9 pots. Subscript numerals represent treatments in cwt/acre.

[2] Sample 30 April 1957 taken before sowing, after addition of lime; sample 10 June 1957 at completion of germination; sample 19 November 1957 at harvest.

[3] Angle $=$ arcsin $\sqrt{\text{percentage}}$.

N_3P_1 (N_0P_6, 39.3%; N_0P_1, 22.6%; N_3P_6, 12.9%; N_3P_1, 1.9%), but the opposite was true of the nitrogen content of the roots (N_0P_6, 1.14%; N_0P_1, 1.36%; N_3P_6, 2.34%; N_3P_1, 2.58%). Here again, the higher the nitrogen concentration in the roots the poorer the mycorrhiza development.

Experiment 2. To provide further information on the nitrogen-mycorrhiza relationship, this experiment was designed to test the effect of calcium carbonate and partial sterilization of the soil on nitrification of ammonium nitrate. A factorial arrangement of the following treatments was used:

- Soil, unsterilized, partially sterilized with 4 percent formalin, or sterilized and then inoculated with fresh soil;
- Calcium carbonate, nil or 16 cwt per acre;
- Ammonium nitrate, nil or 3 cwt per acre.

The basal fertilizer was potassium dihydrogen phosphate at a rate equivalent in phosphorus content to 6 cwt per acre of superphosphate.

The trial was sown on April 30 and harvested on December 3, 1957. The results are given in Table 2. There was little nitrification of ammonium nitrate in the absence of lime. The low yield of shoots at the highest level of soil nitrate (N_3Ca_{16}) is due partly to the fact that this treatment caused heavy mortality among the newly germinated seedlings: the number of seedlings per pot in this treatment averaged 17, whereas yields for other treatments are based on 20 seedlings per pot. The differential response of shoots and roots resulted in an increase in shoot/root ratio as the soil nitrate level rose, the usual effect (Meyer and Anderson 1952, p. 691). The nitrogen concentration in both shoots and roots was closely correlated with the level of nitrate in the soil, hence the relationship between plant nitrogen and yield is similar to that between soil nitrate and yield.

The addition of ammonium nitrate caused a fall in soil pH of about half a unit, while calcium carbonate increased pH by twice this amount. In spite of their contrasting effects on soil reaction, both ammonium nitrate and calcium carbonate reduced mycorrhiza development. An increase in the level of soil nitrate, whether brought about by adding the nitrate ion or by liming, was followed by decreased production of mycorrhizae.

Relationship between mycorrhiza development and carbohydrate status of roots. In order to test Björkman's hypothesis that the formation of mycorrhizae is conditioned by the presence of soluble sugars in the roots, seedling roots from 17 pots were analyzed for reducing sugars. The values obtained showed no correlation with mycorrhiza percent ($r = 0.13$), which result contrasts with that obtained by Björkman (1944).

Björkman's hypothesis is very similar to many others put forward to explain the formation of root nodules in legumes. These have been discussed in a monograph by Wilson (1940), who pointed out that although there was a general correlation between the level of carbohydrate in the roots of legumes and the development of nodules, the relationship did not always hold; however, if the nitrogen supply was also considered, much of the data apparently at variance with the simple carbohydrate hypothesis could be reconciled. In other words, nodule development could be more readily explained in terms of the carbohydrate-nitrogen relationship than in terms of the carbohydrate supply alone. The carbohydrate-nitrogen relationship can be expressed in a number of ways, the simplest and most widely used indicator being the percentage of nitrogen, which in general varies inversely with the carbohydrate/nitrogen ratio.

The data in Tables 1 and 2 show that an inverse relationship between mycorrhiza development and the percentage of total nitrogen in the roots of pine seedlings does indeed exist. A clearer picture of this relationship was obtained by determining the regression of mycorrhiza percent on the percentage of nitrogen in the roots of those seedlings analyzed for reducing sugars. A second degree polynomial equation was fitted to the data (Fig. 1), and the curvilinearity of the regression was found to be significant at the 99 percent level. As might be inferred from this result, a positive correlation exists between mycorrhiza percent and the ratio of soluble carbohydrate (reducing sugars) to total nitrogen; here the correlation is linear (Fig. 2).

Discussion

In this study of the relationship between nutrient supply and mycorrhiza development, the effect of nitrogen was paramount. Even the increase in mycorrhiza

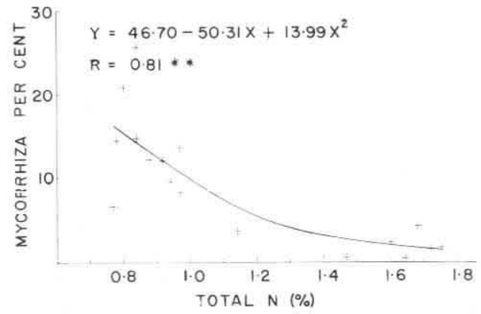

FIGURE 1. *Experiment 2: Relationship between mycorrhiza development and total nitrogen content of seedling roots of caribbean pine* (P. caribaea *Mor.*).

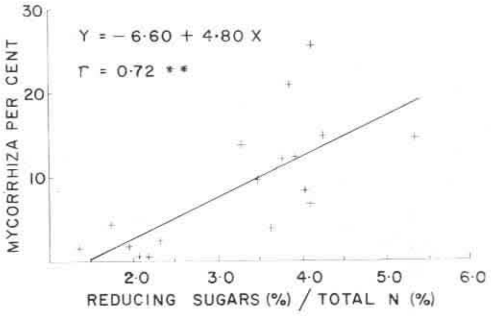

FIGURE 2. *Experiment 2: Relationship between mycorrhiza development and the carbohydrate/nitrogen ratio in seedling roots of caribbean pine* (P. caribaea *Mor.*).

percent brought about by phosphorus in Experiment 2 can be interpreted in terms of its effect on the nitrogen status of the host, since adding phosphorus decreased the nitrogen concentration in seedling roots. The effect of change in soil reaction on mycorrhiza percent brought about by liming was clearly due to the stimulation of nitrification, and not to an increase in soil reaction per se. It is significant that mycorrhizae developed more profusely at pH 7.5, where the soil nitrate level was low, than at pH 5.8, where a much greater amount of nitrate was present (Table 2). This result supports Björkman's findings (Björkman 1941) that mycorrhizae developed as well on seedlings growing in soils of pH 7.2 to 7.5 as on seedlings raised in soils of pH 4.5 to 5.0.

These observations call for modification of the widely held premise that sparse development of mycorrhizae on trees growing in neutral or alkaline soils is due to the acidophilic nature of the mycorrhizal fungi. Whatever the cause of poor mycorrhiza development in such soils, the direct effect of hydrogen ion concentration on the growth of the fungus probably plays a minor role. The indirect effect of soil pH on the availability of soil nitrogen would seem to be more important. It should not be inferred, however, that a high level of available nitrogen is the only factor involved in the inhibition of mycorrhiza formation in alkaline soils (see Björkman 1941).

The conclusion that alkalinity per se does not affect mycorrhiza development seems at first sight incompatible with the established fact that mycorrhizal fungi in pure culture grow best on acid media. However, as emphasized by Voigt (1962), the exchange sites of living root surfaces are continually being recharged with hydrogen ions. This dominance of hydrogen ions at the root-soil interface presumably acts as a buffer against extreme changes in soil pH, and provides a rationale for mycorrhiza formation in neutral and alkaline soils.

As recognized by Björkman (1942), the reactions of mycorrhizae of pines to the addition of combined nitrogen are analogous to those of nitrogen-fixing nodules of legumes. The inverse relationship reported here between mycorrhiza percent and the nitrogen content of seedling roots is taken as indirect evidence in support of Björkman's hypothesis that mycorrhiza formation depends on the availability of soluble carbohydrate in the roots. This may be true even if no direct correlation exists between the soluble carbohydrate level and the degree of mycorrhizal infection, since—as for root-nodule development in legumes—mycorrhiza development in pine seedlings may be more closely related to the carbohydrate/total nitrogen ratio in the roots than to the carbohydrate status alone.

The "available carbohydrate" hypothesis is no longer considered an adequate explanation of the phenomenon of nitrate inhibition of nodulation in legumes (Raggio and Raggio 1962). Likewise, mycorrhiza formation in pine seedlings is no longer thought to be determined entirely by the soluble carbohydrate status of their roots (Slankis 1961), even though such carbohydrate may be the principal energy source for the fungal symbiont. The production of the so-called "M-factor," which stimulates the growth of many mycorrhizal fungi in pure culture, is affected by the nutritional status of the host (Melin 1962); whether this will account for all variations in mycorrhiza development caused by altering the nutrient supply remains to be seen. The effect of environment on the production of root exudates is complex, since not only the amount of exudate, but also the relative proportions of its constituents, are affected by a change in the growing conditions of the host (Rovira 1959). Hence it may be some years before a full understanding of the relationship between the carbohydrate/nitrogen ratio and mycorrhiza development is reached. In the meantime however, it will remain a useful concept for interpreting the effect of cultural treatments on the mycorrhizal status of pine seedlings.

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